

STUDIES ON CERULOPLASMIN AND DIAMINE OXIDASE

KINETICS AND OTHER CHARACTERISTICS

BY

ULF NYLÉN

LUND 1974

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TO MOTHER AND SISTER

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The names of those to the following publications

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TO MAGGIE AND SUSANNE

The thesis is based on the following publications:

1. The Effect of Diethylpyrocarbonate on some Physical and Chemical Properties of Ceruloplasmin.
Ulf Nylén and Gösta Pettersson. Eur. J. Biochem 27 (1972) 578-584.
2. Kinetics of the Interaction between Ceruloplasmin and Reducing Substrates.
Per-Olof Gunnarsson, Ulf Nylén and Gösta Pettersson. Eur. J. Biochem 37 (1973) 41-46.
3. Effect on pH on Electron-Paramagnetic-Resonance Spectra of Ceruloplasmin.
Per-Olof Gunnarsson, Ulf Nylén and Gösta Pettersson. Eur. J. Biochem 37 (1973) 47-50.
4. Inhibition of Ceruloplasmin by Inorganic Anions.
Per-Olof Gunnarsson, Ulf Nylén and Gösta Pettersson. Eur. J. Biochem 27 (1972) 572-577.
5. Kinetics and Other Characteristics of Diamine Oxidase of Pea Seedlings.
Ulf Nylén and Peter Szybek. Acta Chem. Scand (in press).

INTRODUCTION

Photosynthesis and biological reactions are antipodes to each other within the carbon cycle. These two processes are equally necessary for the continuity of life. Through the photosynthetic reactions CO_2 and H_2O are combined, with the help of energy generated by the sun, to form carbohydrates and molecular oxygen. The carbohydrates are then used by various organisms both as raw material and as an energy source. The energy trapped in carbohydrates and similar molecules is made available through stepwise enzymatic degradation. Since oxidative reactions are involved in the release of this energy, the presence of suitable electron acceptors is required. A variety of molecules can serve as acceptors, but in most organisms molecular oxygen functions as the ultimate recipient of electrons. At physiological temperatures, however, oxidative processes involving molecular oxygen are comparatively slow and would not take place at a rate necessary to maintain biological functions unless the reactions were catalyzed. The knowledge of how molecular oxygen is catalytically reduced is of vital importance because it forms the basis for an understanding of aerobic life.

In eukaryotic cells cytochrome-C-oxidase is the most important enzyme catalyzing the reduction of molecular oxygen. It interacts directly with oxygen and is thus the ultimate component in the mitochondrial electron-transport chain. Cytochrome-C-oxidase catalyzes the conversion of both atoms of molecular oxygen into water. Three other enzymes are known to catalyze reactions involving a similar reduction of oxygen to water, namely ascorbate

oxidase of higher plants, laccase from various plant sources and ceruloplasmin from mammalian blood plasma. All these enzymes are metalloproteins containing at least four copper atoms, which might reflect some fundamental requirement for the presence of at least four transition metal ions per enzyme molecule catalyzing the reduction of molecular oxygen into water.

The most important of the four enzymes is undoubtedly cytochrome-C-oxidase, but because of the complexity of the enzyme, which contains six subunits and a large amount of lipid, more information concerning the general features of the mechanism of action of all four enzymes may be provided by studies on the less complex enzymes, ceruloplasmin and laccase.

In addition to the above four enzymes, which catalyze the reduction of oxygen to water, there are a variety of enzymes catalyzing oxidative processes involving molecular oxygen leading to the formation of hydrogen peroxide and/or the incorporation of oxygen into the carbon skeleton of the substrate. Although the cofactors of these enzymes differ the enzymes very often contain transition metals such as iron or copper.

Under physiological conditions and in the absence of reducing substrates the oxidation state of the metal ions in different copper-containing oxidases may be +1 but is more often +2. During the catalytic reactions, Cu^{2+} in some enzymes changes its valence state in response to reducing substrates^{1,2}, while the oxidation state of copper in other enzymes seems to be unaffected^{3,4}.

In the case of ceruloplasmin, which contains six or possibly seven copper atoms^{5,6}, 45% of the copper is detectable by EPR

and magnetic susceptibility measurements⁷⁻⁹, and is thus in the form of cupric copper. The rest of the copper was long believed to be cuprous. However, after the observation that all copper ions in ceruloplasmin can accept electrons⁹, this remaining copper has been proposed to constitute tightly coupled pairs of cupric ions¹⁰. The copper detected by EPR can be divided into two distinct classes: type 1 copper which has unusually narrow hyperfine splitting and is responsible for the blue colour of the protein and type 2 copper which has EPR-parameters more like low molecular weight copper complexes.

In kinetic studies of ceruloplasmin, dimethyl-p-phenylenediamine (DPD) has often been used as a substrate. Earlier reports of the oxidation of DPD by the enzyme gave kinetics which indicated the presence of two active sites^{11,12}. Later it was shown that the initial product (DPD⁺) was also a good substrate¹³. However, DPD⁺ dismutates into DPD and DPD²⁺^{14,15}, and after correction for kinetic effects due to the dismutation process Pettersson¹⁵ found that the oxidation of DPD and DPD⁺ by ceruloplasmin follows a Michaelis - Menten type of rate equation. This elucidation of the kinetics of the reaction made it possible to obtain information about the catalytic mechanism of the enzyme from inhibitor studies.

In the present dissertation an investigation of the function of pea seedling diamine oxidase is included. This enzyme was chosen for further studies on oxidases since the enzyme contains only one atom of copper¹⁶ (Paramagnetic Cu²⁺), its affinity for oxygen is very high (almost comparable with the affinity between oxygen and ceruloplasmin) and the absorption spectra of the oxidized and reduced enzyme

show some characteristic absorption bands which were thought to be valuable tools for evaluation of the kinetics.

CERULOPLASMIN

The catalytic process of oxidation of different substrates by ceruloplasmin and other related oxidases is very complicated. It is not yet clear how the copper atoms in ceruloplasmin participate in the catalytic reactions, and even less is known about the binding situation of different copper atoms in the enzyme. It has been proposed that histidine residues in the protein may be ligands to one or more of the copper atoms^{17,18}. Investigation by Bannister and Wood¹⁹ showed that when ceruloplasmin was photo-oxidized in the presence of methylene-blue the enzyme was strongly inhibited. Most of the inhibition was attributed to modification of the histidine residues which might be ligands to the copper atoms in the enzyme. However, photo-oxidation of ceruloplasmin leads to an extensive loss of copper as well as destruction of histidine and tryptophan residues¹⁹. This might indicate that inactivation of ceruloplasmin during photo-oxidation is related to factors other than the destruction of histidine residues, and it seems evident that photo-oxidation is of insufficient specificity to be used for studying the effect of modification of imidazole groups in ceruloplasmin.

Diethylpyrocarbonate, on the other hand, has been reported to react specifically with histidine residues in proteins at pH 6²⁰. Ceruloplasmin modified by this reagent did not show any change in the absorption spectrum in the visible region when less than 50% of the histidine moieties were carbethoxylated, indicating that the two types 1 Cu²⁺ were not affected by the modification. The activity of the enzyme, however, decreased continuously with increased modification of histidine residues. Electron paramagnetic resonance

spectra of the enzyme showed that carbethoxylation of histidine residues had a pronounced effect on the type 2 Cu^{2+} signal. Treatment of ceruloplasmin with diethylpyrocarbonate caused a decrease in the amplitude of the original low-field line of type 2 copper centered around 2660 gauss. Concomitantly, the modification causes new lines to appear at about 2600 and 2750 gauss. The amplitude of these new fields are correlated with the extent of carbethoxylation of imidazole residues in the enzyme. Thus the binding situation of type 2 Cu^{2+} is evidently changed to about the same extent as the decrease in enzyme activity. However, the present results do not provide evidence that EPR spectral effects and loss of oxidase activity are necessarily attributable to carbethoxylation of the same histidine residue. Investigation of carbethoxylated ceruloplasmin showed that there was a small shift in the EPR signal of type 1 Cu^{2+} after the pH was changed. A similar pH-dependent change of the EPR spectrum could be observed with native enzyme. Since the rate at which type 1 Cu^{2+} is reduced in the presence of substrates was later found to be strongly pH dependent (Paper 2), further investigation of the effect of pH on type 1 Cu^{2+} in native ceruloplasmin was considered to be of interest (Paper 3).

The kinetic studies in paper 2 were performed with ascorbate as substrate in the presence of high concentrations of EDTA. The chelating agent EDTA was used to prevent the oxidation of ascorbate via a ceruloplasmin-iron coupled system. There has been much controversy concerning the substrate specificity of ceruloplasmin, and ascorbate was earlier reported to be exclusively oxidized via a shuttle involving Fe^{2+} ²¹. In paper 2, however, evidence is

given that ascorbate is a true substrate of ceruloplasmin, which confirms results obtained by Curzon and Young²².

Transient-kinetic investigations with ascorbate showed that decolorization of the 610 nm chromophore is due to a rate-determining second-order interaction between enzyme and substrate. Furthermore, this decolorization was found to be independent of the oxidation state of the 340 nm chromophore (non-paramagnetic Cu).

It has been suggested earlier that the kinetics of ceruloplasmin - catalyzed reactions indicate the formation of an enzyme-substrate complex²³. However, kinetic studies of the enzymatic oxidation of mixtures of phenylenediamine derivatives have failed to provide evidence of significant competition for a common substrate-binding site (Paper 4, 24, 25). Evidence pointing to the same conclusion is reported in paper 2, where it is shown that the second-order rate constant for reduction of ceruloplasmin by various substrates is linearly correlated to the reciprocal value of the corresponding K_m -values, and agrees in magnitude with the steady-state kinetic quotient V/K_m . This means, in view of the substrate independence of the maximum activity²⁵⁻²⁷, that differences in the rate of enzymatic oxidation of various substrates can be attributed mainly to differences in the rate of the initial reduction of the 610 nm chromophore, which are in turn reflected by a corresponding variation of the K_m -values. Hence it may be concluded that the established exponential of the substrate²⁴ is derived from a corresponding variation in the rate of the initial reduction of the enzyme. This indicates that differences in the activation energy for the reduction of type 1 Cu^{2+} are mainly determined by the energy required for ionization of the reducing substrate. In paper 3 it is shown that a

significant shift in one of the type 1 Cu^{2+} signals in human ceruloplasmin occurs within the same pH interval as the decrease in the rate of oxidation of ascorbate. The position of the hyperfine lines in EPR spectra of porcine ceruloplasmin, on the other hand, were found to be unaffected by variation of pH. As the kinetic behaviour at different pH values of ceruloplasmin from man and pig is quite similar, it may be concluded that there is no general relationship between pH-dependent variations in the rate of reduction and pH-dependent changes of EPR spectral properties. Calculation of the relative intensity of the type 2 Cu^{2+} signal in EPR spectra of human ceruloplasmin showed that type 2 copper accounts for about one third of the total amount of paramagnetic copper. Since recent evaluations of the molecular weight of ceruloplasmin indicate a total copper content of 6 or possibly 7 atoms per enzyme molecule^{5,6} and since about half of the copper is not detectable by EPR measurements⁸, the above experimental data suggest that ceruloplasmin contains only one type 2 Cu^{2+} and two type 1 Cu^{2+} . In view of these data, the EPR spectral line at 2830 gauss, which is unaffected by variation of pH, most likely represents a main contribution from the first low-field hyperfine line of a second form of type 1 copper rather than from the second low-field hyperfine line of type 2 copper as was suggested earlier from EPR investigations on less homogenous commercial preparations of ceruloplasmin²⁸. Due to the extensive overlapping of copper signals of different types, the corresponding EPR parameters cannot be unequivocally determined from spectra. However, the simulated spectra in paper 3 show that good agreement between simulated and experimental spectra can be obtained from the assumption that there is one type 2 Cu^{2+} and two non-identical type 1 Cu^{2+} in ceruloplasmin.

Paper 4 presents an investigation of the steady-state rate behaviour of ceruloplasmin in the presence of inhibitory inorganic anions. Analysis of the kinetics of inhibition showed that halide ions interact with both the oxidized and the reduced enzyme. However, the inhibition was mainly caused by the formation of a complex between the halide ions and the reduced form of the enzyme. Complex formation between the oxidized enzyme and azide or cyanate was found to be kinetically insignificant at low concentrations of the inhibitors. Furthermore, the present investigation clearly shows that the inhibition of ceruloplasmin by azide occurs through the formation of a very strong complex between the reduced form of the enzyme and azide (stability constant $\geq 500 \text{ mM}^{-1}$). No evidence was obtained for any kinetically significant formation of ternary enzyme-substrate-inhibitor complexes with any of the inhibitory anions tested. The apparent competition between substrate and some inorganic anions and carboxylic acids²⁵ might indicate either that inhibitors compete with substrates for oxidized enzyme, or that formation of enzyme-substrate is kinetically insignificant per se. The lack of common structural similarities between substrates, inhibitory inorganic anions, and carboxylic acids, as well as the established kinetic differences between the two groups of inhibitors²⁵, appears to exclude the possibility that substrates and all inhibitors compete for the same enzymatic site. Thus the present results, together with those concerning the behaviour of the enzyme when reduced by different substrates (Paper 2), strongly indicate that the formation of enzyme-substrate complexes is kinetically insignificant in the absence of inhibitors also.

PEA SEEDLING DIAMINE OXIDASE (Paper 5)

The catalytic function of different amine oxidases from different species are of great biological interest, since most of their natural substrates belong to the group of biogenic amines.

Diamine oxidase of pea seedling is of particular interest because of its unusually high specific activity and unusually high affinity for oxygen. Furthermore, the enzyme has been reported to contain only one mole of copper per mole of protein¹⁶.

The catalytic mechanism for pea seedling diamine oxidase is not well understood, but the present investigation provides evidence that the enzymatic reactions conform to a ping-pong mechanism. Contrary to other amine oxidases²⁹, the rate determining step in catalysis by diamine oxidases of pea seedlings is independent of oxygen concentration under physiological conditions. More detailed studies concerning transient-state kinetics failed because most of the transient-state reactions happened within the dead-time of the stopped-flow apparatus.

There is strong evidence that the activity of several copper containing amine oxidases is dependent upon the presence of pyridoxal phosphate as a prosthetic group^{30,31}. Titration of pea seedling diamine oxidase with phenylhydrazine showed that this enzyme contains one mole of carbonyl functional group per mole of enzyme (Paper 5). In order to see if this group is pyridoxal phosphate the following experiment was undertaken. The enzyme was reacted with ¹⁴C-putrescine in an oxygen-free solution, reduced with NaBH₄ and dialyzed against a large excess of water. This treatment led to a significant incorporation of radioactivity into the protein. The enzyme was hydrolyzed for

22 hours in 6M HCl at 110°C. However, none of the radioactive spots found after paper electrophoresis of the hydrolysate at pH 3.6 was identical with reference substances treated in the same way. As putrescine was readily incorporated into the enzyme, these results may be taken as an indication that the carbonyl group in diamine oxidase of pea seedlings might be other than pyridoxal phosphate.

CONCLUDING REMARKS

Papers 1 to 5 make a small contribution to the understanding of some reaction steps in the catalytic mechanism of two copper-containing oxidases. Thus the rate of oxidation of various substrates by ceruloplasmin seems to be attributable mainly to differences in the ionization potential of reducing substrates. The reducing substrates do not seem to form kinetically significant complexes with the enzyme. Inhibitors like azide are in low concentration bound mainly to the reduced form of the enzyme. Furthermore, it seems likely that some conformational change may be necessary before the inhibitors are bound, and that a similar situation may exist concerning the binding of oxygen. Kinetic and EPR measurements on carbethoxylated ceruloplasmin gave a weak indication that histidine might be involved in the binding of type 2 copper, but otherwise very little is known concerning the arrangement of the metal centers, the electron distribution within the enzyme and how the high activation energy for oxygen is overcome. These and some related problems must be resolved before a proper mechanism for ceruloplasmin and other copper-containing oxidases can be given.

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